Journal of Medical Virology 61:251–258 (2000)

Multiplex Polymerase Chain Reaction for the Evaluation of Cytomegalovirus DNA Load in Organ Transplant Recipients

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Because of the considerable impact of human cytomegalovirus (HCMV) infection, sensitive, specific, and standardized methods are required for rapid and accurate evaluation of viral load in monitoring transplant recipients. The aim of the present study was to evaluate the usefulness of a multiplex polymerase chain reaction (PCR) for the coamplification of HCMV-DNA and β-globin genomic sequence in polymorphonuclear leukocytes (PMNL). Analysis and quantification of PCR products were carried out by a DNA enzyme immunoassay (DEIA), which is based on the hybridization of amplified DNA with a single-stranded DNA probe, which coats microtitre wells. Colorimetric detection of the DNA-antibody complex was carried out and optical density (O.D.) was recorded at 450/630 nm. To quantify HCMV/DNA load, a standard curve to which samples O.D. refer was obtained by amplifying serial dilutions of recombinant pGEM-3Z plasmid DNA containing a genomic fragment of glycoprotein B. 340 PMNL specimens from 102 solid organ recipients were tested for the detection of pp65 antigen and HCMV-DNA. The results showed a good correlation between viral load and clinical symptoms of HCMV infection; high specificity and predictive values for HCMV disease were found by PCR, using a cut-off limit of 10^3 genomic copies per 2×10^5 PMNL. These findings indicate that the system described is an efficient and reproducible diagnostic method easy to apply for routine diagnosis and therapeutic monitoring of transplanted patients. ***J. Med. Virol. 61:251–258, 2000.***

KEY WORDS: human cytomegalovirus; transplantation; quantitative PCR; DNA enzyme immunoassay

INTRODUCTION

Cytomegalovirus (CMV) is a ubiquitous member of the Herpesviridae family, which infects the majority of the population and can establish lifelong persistence after primary infection of the host. Although human cytomegalovirus (HCMV) infection is common and in healthy humans is usually subclinical and occurs asymptomatically, in the immunocompromised host, both primary and recurrent infections can directly induce serious illness, representing the major cause of morbidity and mortality despite improved treatment [Ho, 1993].

The incidence of HCMV infection in transplant patients ranges from 23% to 85%. The major consequences include either localized or disseminated HCMV disease [Kanj et al., 1996]. Superinfection with opportunistic pathogens can result from host defects caused by the virus. In addition, HCMV is considered a risk factor for graft rejection, inducing the manifestation of histocompatibility antigens in infected cells [Grundy et al., 1988]. However, the symptoms related to HCMV infection are often difficult to discriminate from clinical conditions associated with rejection crises [von Willebrand et al., 1986; Pasternack et al., 1990].

The wide range of HCMV complications emphasizes the need for diagnostic techniques, which can give information rapidly and reliably about the status of infection, in an effort to guide specific antiviral therapy and to allow judicious management of immunosuppression and monitoring of graft function.

A specific and rapid assay for diagnosis and monitoring of an active HCMV infection is based on the detection of the lower matrix protein pp65 in peripheral blood leukocytes [Grefte et al., 1991; Ghisetti et al., 1996]. The number of positive cells has been shown to be related to active infection [Gerna et al., 1992].

Recently, HCMV diagnosis and monitoring have been greatly improved, detecting viral nucleic acid in peripheral blood using the polymerase chain reaction (PCR) [Boland et al., 1992; Zipeto et al., 1992; Drouet et

Grant sponsor: Facolta

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Accepted 1 November 1999

al., 1993]. However, the high sensitivity of conventional qualitative amplification methods represents a limit in the differentiation between replicative and latent infection and in the lack of positive predictive values [Delgado et al., 1992; Bitsch et al., 1993a; Weber et al., 1994; Kanj et al., 1996].

In fact, in immunosuppressed patients, a considerable percentage of positive PCR results can be found without further evidence of HCMV symptomatic disease. PCR could remain positive for a long time, even after successful treatment. For this reason, the application of the reverse transcription (RT)-PCR method for the study of HCMV transcripts for Immediate Early (IE) and Late proteins in peripheral blood cells as a measure of active viral replicative status has been suggested [Bitsch et al., 1993b; Gozlan et al., 1993; Randawa et al., 1994]. Nevertheless, it has been reported that late transcript detection seems to be indicated more for the follow-up evaluation of symptomatic patients during antiviral therapy than for early diagnosis [Meyer-Konig et al., 1995; Gaeta et al., 1997].

Assuming that the viral genomic load is strictly related to the severity of infection, several investigators have suggested procedures to quantify HCMV-DNA in peripheral blood and to minimize unwanted positive results due to latent viruses [Zipeto et al., 1993; Drouet et al., 1995; Fox et al., 1995; Payan et al., 1997]. Strategies designed to quantify the HCMV DNA load, in order to increase the PCR specificity have therefore been developed. Recent approaches have included semiquantitative evaluations based on limiting dilution of samples or comparison of amplicon signal intensity with defined serial dilutions of an external standard [Schafer and Laufs, 1996].

Good results have been also obtained by competitive PCR protocols based on the coamplification of a positive internal control, which shares the same primers with HCMV sequence target [Chan et al., 1994; Fox et al., 1995]. However, the assays described are not of immediate clinical use, due to expensive and cumbersome detection procedures and low sensitivity [Boivin et al., 1997].

During the past few years, promising results have been obtained by the use of DNA enzyme immunoassays (DEIA) for the analysis of amplification products. This method is based on the hybridization of amplified DNA with a single-stranded DNA probe, which coats the wall of microtitre plate wells. The detection of hybrids is achieved by means of an anti-double-stranded DNA antibody, by means of a colorimetric assay resembling a common enzyme immunoassay. Several investigators have reported that the enzymatic detection of amplicons has shown great sensitivity, specificity, and reproducibility and that it may be a good method for quantitative analysis of genomic loads [Mantero et al., 1991; Allen et al., 1995]. The present report examines an assay system for quantitative evaluation of HCMV genomic load in clinical samples from solid organ transplant patients; the results were compared with a quantitative study of pp65 antigen and with the clinical condition of patients.

MATERIALS AND METHODS

Patient Population

The study involved 102 patients who received solid organ transplants at the Transplant Unit of Policlinico Umberto I Hospital in Rome from January 1997 to January 1998. Forty-four patients underwent kidney transplants, 31 lung, 23 liver, and 4 heart transplants. After transplant all patients received prophylactic therapy against viral infection. For kidney and liver transplants, patients were treated with oral administration of acyclovir for 1 month (400 mg/die 3 times daily); for lung transplants, intravenous ganciclovir was used for 3 weeks (10 mg/kg/die) and acyclovir continuously (800 mg/die). The dosage of immunosuppression and the treatment of rejection episodes were established for each patient group according to specific protocols that took into consideration the patient's clinical condition. Venous blood samples were collected weekly during the patients' in hospital recovery period. The length of HCMV routine surveillance was dictated by the type of transplant and was respectively 20–25 days for kidney and liver, and 25–30 days for heart and lung transplants. Patients were considered to be infected with HCMV by the presence of pp65 antigen and/or HCMV DNA in PMNL. HCMV infection was defined as asymptomatic when no clinical symptoms or laboratory abnormalities were detected. HCMV disease was defined as active infection when antigenemia and/or PCR positivity in association with one or more of the following symptoms: fever, leukopenia, thrombocytopenia, and/or evidence of organ involvement.

Patients with HCMV symptomatic infection were treated with ganciclovir at the dosage of 10 mg/kg/die by the intravenous route for 2 weeks. Hyperimmune globulin therapy was added in those recipients who had prolonged HCMV infection.

Polymorphonuclear Leukocyte Sample Preparation

Polymorphonuclear leukocytes (PMNL) were isolated from EDTA-treated venous blood by sedimentation. Briefly, 5 ml of blood was allowed to incubate at 37°C for 20 min. The leukocyte-rich upper layer was harvested and the contaminating erythrocytes were lysed with 0.8% NH_4Cl. Cells were washed twice in phosphate-buffered saline (PBS), counted and adjusted to 5×10^6/ml. An aliquot of PMNL was immediately used for HCMV antigenemia assay and 1 ml was stored at −70°C until use for PCR.

HCMV Antigenemia

Aliquots of 5×10^4 PMNL were prepared on glass slides, then fixed with 5% paraformaldehyde and permeabilized with 0.5% Nonidet P-40 according to the manufacturer's instructions (CMV Ag-Kit, Argene, France). Cells were incubated with a pool of anti-HCMV pk65 monoclonal antibodies and after washing

were treated with fluorescein-conjugated anti-mouse IgG + IgM $F(ab)_2$ fragment. For each sample, a well for conjugate control was used from which the HCMV monoclonal antibodies were omitted. Slides were examined under a fluorescent microscope (Orthoplan, Leitz Wetzlair, Germany) and the results were expressed quantitatively as the number of HCMV antigen positive cells per 2×10^5 PMNL.

PCR Multiplex

PCR for the detection of HCMV DNA was carried out on PMNL; 2×10^6 PMNL of each sample were treated with alkaline thermolysis for DNA extraction and processed according to the manufacturer's instructions (Bioline Diagnostics, Italy). After the extraction procedure, the DNA was resuspended in 100 μl of specific buffer; 10 μl of each sample was subjected to DNA amplification in 90 μl of reaction mixture consisting of 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP), 2.5 U of Taq DNA polymerase (Perkin-Elmer Cetus), and 50 pMol of upstream (P2) and downstream (P4) primers for a sequence of the HCMV glycoprotein B gene [Gaeta et al., 1997]. Moreover, 10 pMol of upstream (GH20) and downstream (PC04) primers was added to the reaction mixture and amplified a 260-bp sequence of the human β-globin gene [Resnick et al., 1990]. The PCR reaction was carried out in an automated thermal cycler (Minicicler, Genenco) for 32 cycles (denaturation 94°C for 1 min, annealing 58°C for 1 min and 30 sec, extension 72°C for 1 min; plus 10 min of extension in the last cycle). As positive control for the extraction and amplification steps, Peu cell line infected with AD169 HCMV strain was used, and the same uninfected cell line and a reaction mixture without DNA were run in each experiment as negative controls. Precautions to avoid carryover and to ensure the validity of the results were strictly observed [Kwok, 1990].

Analysis of PCR Products

Samples of the amplified reaction mixture were tested in three ways. One 20 μl sample was subjected to 2% agarose gel electrophoresis and stained with ethidium bromide; a second 20 μl sample was hybridized with HCMV-specific probe PP [Gaeta et al., 1997], and a third 20 μl sample was hybridized with β-globin specific probe PC03 [Resnick et al., 1990]. In both of these cases, the hybrids were detected by using a DNA enzyme immunoassay (DEIA) (Sorin Biomedica, Saluggia, Italy). This method was based on the hybridization of denatured amplicons with a single-stranded DNA probe coating the wall of microtitre plate wells with a streptavidin-biotin bond. DNA-DNA hybrid was then detected using anti-double-stranded DNA monoclonal antibodies. Briefly, aliquots of PCR products were denatured at 95°C for 10 min and quickly chilled on ice; 20 μl each of controls and specimens was hybridized for 1 h in microtitre plate wells, respectively, coated with 5′ biotinylated probes specific for HCMV and β-globin amplicons. After washing, the wells were incubated for 30 min at room temperature with an anti-ds-DNA antibody solution. The wells were washed again and an enzyme tracer solution, made up of protein A-conjugated with horseradish peroxidase (HRP), was added. After 30 min of incubation at room temperature, the wells were treated with tetramethylbenzidine-hydrogen peroxide solution and kept in the dark for 30 min for color development. The absorbance was measured with a photometer (ETI System Reader-Sorin Biomedica, Saluggia, Italy) at 450/630 nm; for each specimen, the 630 nm was subtracted from the 450-nm absorbance value. The assay was considered to be valid if, after subtraction of substrate blank, the mean negative assay control was <0.05, and the mean positive assay control was >0.4.

Quantitative Analysis

In order to make a quantitative evaluation of HCMV DNAemia, a standard curve to which O.D. samples refer was generated. A 301-bp genomic fragment of glycoprotein B gene, including the 229-bp sequence amplified by P2 and P4 primers, was inserted into pGEM-3Z plasmid. The recombinant plasmid was then used to transform competent cells. Plasmid DNA was extracted using the alkaline lysis procedure, and serial dilutions were prepared; 10 μl of each dilution, containing 10^{-1} to 10^8 plasmid genomic copies, was amplified using the couple of primers for HCMV glycoprotein B. The PCR products for each concentration were tested using either gel electrophoresis and Southern blot or DEIA.

For Southern blot, amplified products were transferred from gel to nylon membrane (Hybond N^+, Amersham International, UK) and hybridized with PP internal probe digoxigenine labeled at 3′ end. Hybrids were detected by enzyme-linked immunoassay using an antibody-conjugate (anti-digoxigenine alkaline phosphatase conjugate) visualized by color reaction with 5′-bromo-4-chloro-3-indolyl-phosphate (X-phosphate) and nitroblue tetrazolium (NBT) salt (Boehringer Mannheim, Germany).

To calculate the HCMV genomic copies related to the number of processed cells in clinical specimens, the amplified sample O.D. were referred to a standard curve created by plotting the O.D. mean value relative to three different experiments and the co-respective standard deviation against the number of input HCMV genomic copies (Fig.1).

Statistical Analysis

PCR results were expressed as mean value ±SD. Significance was expressed and evaluated by Student's *t*-test and *P* values of <0.05 were considered significant. The determination of sensitivity, specificity, and predictive values used the standard definition [Griner et al., 1981]. Correlation between quantitative PCR and antigenemia was done using the Pearson correlation test.

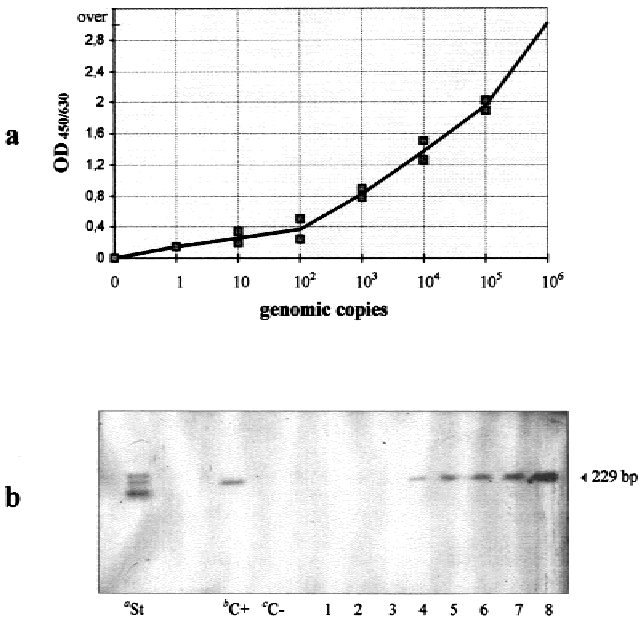

Fig. 1. Serial dilutions of recombinant PGEM3Z plasmid including a HCMV glycoprotein B gene sequence. **Section a** refers to the standard curve obtained by plotting the OD mean values and SD relative to three different trials. **Section b** shows Southern blot hybridized with digoxigenine labelled at 3′ end probe. Lines 1 to 8 correspond to the amplicons of recombinant plasmid dilutions from 10^{-1} to 10^6 genomic copies. [a], Standard DNA molecular weight; [b], PCR positive control – HCMV AD169; [c], PCR negative control.

RESULTS

Patients and Incidence of HCMV Disease

A total of 340 PMNL specimens from 102 solid organ recipients were tested for the detection of pp65 antigen and HCMV DNA. HCMV infection, determined by the positivity to antigenemia and/or PCR, was detected in 53 of 102 (51.9%) patients included in the study. The incidence of HCMV was 61.3% of lung recipients, 52.3% of kidney recipients, and 30.4% of liver recipients; it occurred in 100% of heart transplant recipients.

Of 53 infected patients, 28 patients (52.8%) experienced at least one episode of symptomatic HCMV disease. The most frequent clinical signs were fever, leukopenia, a rise of creatinemia and, with regard to organ invasion, the lung was found to be the most affected organ. Twenty-five infected patients (47.2%) with PCR and/or antigenemia remained free of clinical symptoms; no evidence of HCMV infection by antigenemia or by PCR was observed in 49 of 102 (48.1%) patients studied (Table I).

TABLE I. Incidence of HCMV Infection Among Solid Organ Transplant Recipients

	HCMV infection		
	Symptomatic	Asymptomatic	No infection
Lung	7	12	12
Kidney	15	8	21
Liver	2	5	16
Heart	4	0	0

HCMV, human cytomegalovirus.

Standardization of Multiplex PCR

In order to avoid false-negative results, the model system was standardized, including an internal control that clearly specified the variability in the extraction and amplification steps. For this purpose each DNA sample extracted was coamplified for HCMV glycoprotein B gene and for a sequence of human β-globin. Several PCR conditions concerning the primer pairs' concentration and cycling parameters were investigated in order to perfect the procedure, minimizing interference in the simultaneous amplification outcome, and to increase sensitivity to inhibitors or differences in DNA recovery.

In this investigation, 10 pM of upstream and downstream primers in the reaction mixture was chosen for amplification of the human β-globin gene. The low concentration of β-globin primers was designed to compensate for the greater copy number in PMNL samples relative to that expected for HCMV sequences. An adjustment was also made in the detection step of amplified products. Each sample was tested in duplicate by DNA enzyme immunoassay, using both HCMV and β-globin specific-probes for the hybridization. Therefore, a range of acceptance was determined, defined according to a curve made with the O.D. 460/630 obtained by the PCR products of serially diluted concentrations of β-globin sequence (data not shown). Only samples with β-globin O.D. values of 0.650–1.4 were considered acceptable, conforming to, and efficiently performing, the extraction and amplification steps. The value of β-globin O.D. relating to all test samples was within a range of 1.392–0.807, with a mean value of 1.135 ± 0.186. Furthermore, variability of DNA extraction was less than 20%.

Quantification of HCMV Genomic l Load and pp65 Antigenemia Assay

To evaluate HCMV genomic copies by DEIA, the positive cut-off value was considered the greater of (2× mean of NPC control) + 0.100 O.D. unit, where NPC was the negative PCR control. When the cut-off values were examined in relation to the standard curve, they always corresponded to a number of genomic copies lower than 10. Therefore only values of >10 were considered positive. On the basis of virological and clinical data, the results obtained by antigenemia test and PCR were arranged into five groups.

Groups 1 and 2 included samples of patients with clinical signs of HCMV infection; they comprised positive antigenemia results in which the antigenic load was >100 and 20–100 positive cells per 2×10^5 PMNL, respectively. Group 3 comprised the results obtained from specimens corresponding to asymptomatic patients; in the last group, the antigenic load ranged from 4 to 40 positive cells per 2×10^5 PMNL. The qualitative PCR of these groups showed HCMV DNA presence when stained with ethidium bromide. Group 4 was also related to asymptomatic episodes of infection but com-

TABLE II. Distribution of HCMV DNA Load and Quantitative Antigenemia in PMNL Samples from Transplant Patients*

Group	Samples	pp65 (nPos/2 × 10^5 PMNLs)[a]	Qualitative PCR	DEIA (D.O.)	Genomic copies (2 × 10^5 PMNL)
Group 1					
Symptomatic infection	19	>100	pos	Over[b]	>5 × 10^5
Group 2					
Symptomatic infection	67	10–100	pos	1.815 ± 0.728	7 × 10^3–5 × 10^5
Group 3					
Asymptomatic infection	39	4–40	pos	0.825 ± 0.207	8 × 10^2–6 × 10^3
Group 4					
Asymptomatic infection	72	neg	pos	0.571 ± 0.170	10^2–10^3
Group 5					
No infection	143	neg	neg	0.084 ± 0.041	<10

*The groups were assembled according to cytomegalovirus infection signs.
[a]Number of positive PMNL/200,000 to immunofluorescence staining for pp65 antigen.
[b]Absorbance values at 450/630 nm ≥3.00.
HCMV, human cytomegalovirus; PMNL, polymorphonuclear leukocytes; PCR, polymerase chain reaction; DIEA, DNA enzyme immunoassay.

prised samples without antigenemia and positivity to qualitative PCR, whereas in group 5 negative specimens for both tests were included.

The mean and the standard deviation of HCMV genomic copies in PMNL from the five groups are presented in Table II. All samples included in group 1 had a viral load of >5 × 10^5 genomic copies per 2 × 10^5 PMNL. In group 2, genomic copies values ranged from 7 × 10^3 to 5 × 10^5, in group 3 and 4 from 8 × 10^2 to 6 × 10^3 and 10^2–10^3 respectively. In group 5, including negative values, genomic copies were always lower than 10. Statistical analysis showed that the differences in viral DNA load were not significant between group 1 versus group 2 and between group 3 versus group 4, whereas the viral DNA load in symptomatic patients (groups 1 + 2) was significantly higher (P <0.05) than in asymptomatics (groups 3 + 4).

In addition, the sensitivity, specificity, and positive and negative predictive values of PCR assay for the development of HCMV disease were analyzed using quantitative results; the values obtained were 100%, 61.5%, 45.4%, and 100%, respectively (Table III). By using a threshold of >10^3 genomic copies per 2 × 10^5 PMNL as the cut-off, the sensitivity and negative predictive value decreased to 95% and 97%, but the specificity and positive predictive value increased to 87% and 74%, respectively.

Moreover, significant correlation was present between the genomic load and the number of pp65 positive PMNL ($r^2 = 0.75$, P <0.01), except for five samples relative to three patients who underwent lung transplants. In the latter cases, despite the presence of clinical symptoms and high levels of DNAemia (10^4–10^5 genomic copies per 2 × 10^5 PMNL), the antigenic load was less than 10 positive cells per 2 × 10^5 PMNL.

For the antigenemia assay, all symptomatic patients had high levels of antigenic load in association with clinical disease. Six patients had more than 100 pp65 positive cells per 200,000 PMNL during the acute episodes; in the remaining 22 symptomatic patients, the antigenemia values never exceeded the previous value.

Twelve of 25 asymptomatic patients never had antigenemia, while the other 13 asymptomatic patients had an antigenic load of less than 40 positive cells per 200,000 PMNL in one or more specimens. As for the antigenic load, the diagnostic value of antigenemia assay for HCMV disease was also measured in terms of sensitivity, specificity, positive predictive, and negative predictive values. The percentages obtained were 84.2%, 76.3%, 64%, and 90.6%, respectively.

TABLE III. Analysis of Antigenemia and PCR Tests for the Development of HCMV Disease*

	Se (%)	Sp (%)	PPV (%)	NPV (%)
Antigenemia	84.2	76.3	64	90.6
PCR	100	61.5	45.4	100
PCR (cut-off 10^3 genomic copies)	95	87	74	97

PCR, polymerase chain reaction; HCMV, human cytomegalovirus.
*Se, sensitivity, Sp, specificity, and PPV, positive and NPV, negative predictive values are expressed as percentages.

DISCUSSION

It is possible to use different diagnostic tool for HCMV, but the best method has not yet been identified. In transplant patients, it is very important to have a specific and sensitive signal in order to distinguish the HCMV disease from other clinical events that need different therapeutic approaches, such as rejection crisis, that may show similar clinical symptoms at onset. In HCMV infection, it is also essential to differentiate a productive from latent or nonproductive infection, in order to address a rational evaluation of antiviral therapy. It has been shown that the central role in the pathogenesis of HCMV is the virus load; thus, viral titer measurement may differentiate latency from active viral replication. Therefore, many investigators agree that, in making a complete diagnosis of HCMV infection, it is essential to use quantitative approaches [Schafer and Laufs, 1996].

In recent years, a rapid and sensitive method has been developed to identify the HCMV-encoded pp65 antigen in peripheral blood leukocytes; however, there are some crucial steps when carrying out the quantitative antigenemia assay: sensitivity changes notice-

ably with experimental conditions [Boeckh et al., 1994]. In addition, pp65 antigen is not always found in patients with clinical signs of HCMV disease [Weber et al., 1994].

Promising results have been also obtained by the detection of viral nucleic acid in peripheral leukocytes and in plasma [Zipeto et al., 1995]; thus, PCR is considered the most sensitive method, but only when adopted in quantitative systems, when the best results in specificity and predictive value for HCMV disease are obtained [Kuhn et al., 1994; Boivin et al., 1997]. PCR has been suggested for reproducibility in different quantitative or semiquantitative methods for the diagnosis of HCMV infection [Kagle et al., 1992; Boivin et al., 1996]. To date, the use of the different strategies seems to require standardization of all steps to be carried out, including sample processing, DNA amplification and amplicon detection [Roberts et al., 1997; Boeckh and Boivin, 1998; Haberhausen et al., 1998]. A crucial point concerns the control of inhibitors and the amount of DNA obtained from the extraction procedures. Thus, variability in the concentration of DNA in the preparation seems to be an important factor in the outcome of the reaction and its standardization is also essential for sample quantitative analysis [Caballero et al., 1997].

In order to take into consideration all those aspects, a quantitative noncompetitive PCR was designed for the diagnosis of HCMV infection based on a multiplex PCR for the simultaneous detection of HCMV DNA and a sequence of β-globin gene. The amplification of a cellular genetic sequence allowed us to evaluate the efficiency of DNA extraction by PMNL samples and to standardize the amount of DNA present in the amplification mixture. It also tests for the presence of inhibitors. We estimated that in a range of β-globin O.D. from 0.650 to 1.4 the variability of results was low, with an intra-assay and interassay variability of less than 15% and 25%, respectively. To achieve the highest standards of efficiency and specificity in this system, it was essential to optimize the assay conditions, including the concentration of the two couples of primers, taking into account that more cellular DNA is present than viral DNA. For amplification of HCMV genomes, two primers were used for a glycoprotein B gene sequence that had shown good results [Gaeta et al., 1997] and at the same time were suitable for coamplification with β-globin gene sequence.

The use of an enzyme-linked immunoassay for the detection of amplified products was also addressed from several viewpoints. Above all, the hybridization confirms the specificity of the amplicons; it can also increase the sensitivity of the PCR assay compared with that by detection in agarose gel; this allowed the use of a low number of amplification cycles, avoiding saturated conditions of the method. Good reproducibility of the results and the quantitative evaluation of positive samples without further dilutions was achieved.

In the present report, the data on antigenemia and viral genomic load obtained from all samples tested was related to the clinical status of patients, and five possible combinations that included most cases were observed. The results demonstrated good correlation between the viral load and clinical symptoms related to HCMV disease, with a statistical significance ($P < 0.05$) viral load more than 7×10^3 genomic copies per 2×10^5 PMNL corresponded to symptomatic infection [Mutimer et al., 1997; Zaia et al., 1997]. By contrast, the presence of HCMV infection without further clinical signs was underlined by viral DNA load ranging from 10^2 to 6×10^3 genomic copies per 2×10^5 PMNL.

As reported by other investigators, slight symptoms were found to be present even when the antigenic load was less than 100 positive cells/2×10^5 PMNL, and moderate levels of antigenemia were detected in asymptomatic infection. All this is represented in groups 2 and 3 and the overlapping pp65 values could be related to different breakpoints of quantitative antigenemia between diverse transplant populations [van den Berg et al., 1989; The et al., 1992].

Good correlation was found between the values of genomic load and quantitative antigenemia, except in three lung recipients, in whom, despite high levels of HCMV DNA and the presence of clinical evidence of HCMV infection, low levels of pp65 antigen were detected. These data agreed with a report by Weber et al. [1994], in which pp65 antigen detection from peripheral blood leukocytes was not always found in symptomatic HCMV infection. Further, it is possible to correlate the absence of antigenemia detected in some of lung recipients with the effect of antiviral preemptive treatment used for prophylaxis, in agreement with Landry et al. [1995], who reported a significant decline in the number of pp65-positive leukocytes in the presence of anti-HCMV therapy.

Furthermore, when the values of sensitivity, specificity, and positive and negative predictive values for the presence of HCMV disease were compared, better specificity and predictive index were found for the antigenemia assay than for the amplification method, as did Boivin et al. [1998]. But with a cut-off limit of 10^3 genomic copies per 2×10^5 PMNL, besides sensitivity, also specificity and predictive values were higher in PCR results.

The information about circulating viral load and development of HCMV disease, shown by quantitative PCR, could provide the first warning sign of symptomatic infection. In the follow-up evaluation of high-risk patients, the increased viral load, its kinetics, and the constant high levels could provide useful information to clinicians in designing therapeutic protocols.

In conclusion, because of its easy application and good standardization, the method described in the present report is thought to represent a suitable approach for monitoring patients at risk of HCMV infection. However, prospective studies are now required to determine the combination of different sets of primers able to amplify different regions of HCMV genome simultaneously, taking into account the possibility of

variations in the sequences of the viral genomes found in different groups of patients, which could affect the performance of diagnostic PCR [Chow, 1992; Mendez et al., 1998].

ACKNOWLEDGMENTS

We thank Valentina D'Angelo for the excellent technical assistance. This work was partially supported by a grant for Research from "Facoltà 40%—1996" University La Sapienza—Rome, Italy.

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